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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the specification in connection with Application No. 2003904870 for a patent by MINOMIC PTY LTD as filed on 05 September 2003.

WITNESS my hand this
Fourteenth day of September 2004

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Patents Act 1990

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PROVISIONAL SPECIFICATION

Invention Title:

Assay for Diabetes

The invention is described in the following statement:

Technical Field

The present invention relates to new peptides and methods of detecting the presence of the peptides in biological samples to screen for diabetes or identify a predisposition to diabetes in a subject.

Background Art

Every year throughout the world thousands of people die and many thousands more suffer heart and kidney problems, stroke or lose a limb or their vision as a result of Type II diabetes.

Type II diabetes diagnosis and management, for example, is currently hampered by a number of deficiencies. Three areas where better testing is desirable are initial diagnosis, monitoring of blood glucose control, and better monitoring of renal damage.

Urine should potentially be a rich source of biomarkers but for proteomics research the presence of high amounts of salts such as urea have made study difficult. There are a number of other tests being used to diagnose Type II diabetes but none of these is ideal. There are deficiencies in each test that are multifactorial. In many cases, patients do not want to give blood or return for further test and produce multiple samples such as blood and urine. The present invention can cover all three tests with one sample.

In Australia, 15% of 55-65 year olds have Type II diabetes but approximately 50% are undiagnosed due to the reluctance of doctors to order a glucose tolerance test. This test requires a blood sample then a dose of glucose orally followed by another blood sample 2 hours later. A simpler, less invasive test would be commercially very attractive. Furthermore, children who have symptoms of diabetes are usually diagnosed with Type I diabetes. This is of particular concern given the rise in childhood Type II diabetes, and some centers report a misdiagnosis in 25% of cases.

Currently blood glucose control is monitored by the glycosylated haemoglobin test. This the test is complicated by anything that changes the half-life of red cell turnover. A test that shows efficacy in monitoring blood glucose control in the 2-3 day or 1 week period would be highly desirable.

All diabetics should be monitored once a year for renal damage via urine collection. This is not done for around 70% of patients due to compliance issues.

As the number of people with diabetes grows worldwide, the disease takes an ever-increasing proportion of national health care budgets. Without primary prevention, the diabetes epidemic will continue to grow. Even worse, diabetes is projected to become one of the world's main disablers and killers within the next twenty-five years. Immediate action is needed to reduce the onset of diabetes and to introduce more cost-effective diagnostic strategies to reverse this trend.

The present inventors have now identified new peptide markers which are useful in developing non-invasive assays for diabetes.

Disclosure of Invention

In a first aspect, the present invention provides an isolated protein, protein fragment or peptide, detectable in a biological sample of a subject, being indicative of diabetes or a predisposition to diabetes of a subject, the protein, protein fragment or peptide comprises or contains one or more of the following amino acid sequences:

AYIFIDEAHITQALIWLSQR;
KEPSQGTTTFAVTSILR;
LLDNWDSVTSTFSK;
LLIYAVLPTGDVIGDSAK;
LLLQQVSLPELPGEYSMK;
QEPSQGTTTFAVTSILR;
QGLLPVLESFK;
TELRPGETLNVNFLLR;
VFAIPPSFASIFLTK; or
WLQGSQELPR.

Preferably, the protein, protein fragment or peptide is one or more of

AYIFIDEAHITQALIWLSQR;
KEPSQGTTTFAVTSILR;
LLDNWDSVTSTFSK;
LLIYAVLPTGDVIGDSAK;
LLLQQVSLPELPGEYSMK;
QEPSQGTTTFAVTSILR;

QGLLPVLESFK;

TELRPGETLNVNFLLR;

VFAIPPSFASIFLTK; or

WLQGSQELPR.

The peptides according to the invention were obtained by:

(a) concentrating / fractionating urine samples from diabetics and healthy individuals;

(b) separating protein present in the concentrated urine samples; and

(c) identifying protein, protein fragment or peptide present in the urine of diabetics but absent or undetectable in healthy individuals.

The urine was concentrated / fractionated by membrane-base electrophoresis.

The peptides were separated by chromatography .

The peptides were identified by mass spectrometry.

In a second aspect, the present invention provides an isolated antibody directed to a protein, protein fragment or peptide detectable in a biological sample of a subject being indicative of diabetes or a predisposition to diabetes in a subject, the protein, protein fragment or peptide comprises or contains one or more of the following amino acid sequences:

AYIFIDEAHITQALIWLSQR;

KEPSQGTTTFAVTSILR;

LLDNWDSVTSTFSK;

LLIYAVLPTGDVIGDSAK;

LLLQQVSLPELPGEYSMK;

QEPSQGTTTFAVTSILR;

QGLLPVLESFK;

TELRPGETLNVNFLLR;

VFAIPPSFASIFLTK; or

WLQGSQELPR.

Preferably, the protein, protein fragment or peptide is

AYIFIDEAHITQALIWLSQR;

KEPSQGTTTFAVTSILR;

LLDNWDSVTSTFSK;

LLIYAVLPTGDVIGDSAK;

LLLQQVSLPELPGEYSMK;

QEPSQGTTTFAVTSILR;

QGLLPVLESFK;

TELRPGETLNVNFLLR;

VFAIPPSFASIFLTK; or

WLQGSQELPR.

In one preferred from, the antibody is a polyclonal antibody which is derived by immunising mice or other suitable animal with one or more proteins, protein fragments or peptides according to the first aspect of the present invention.

In another preferred form, for the antibody is an isolated monoclonal antibody to one or more proteins, protein fragments or peptides according to the first aspect of the present invention. Methods for developing monoclonal antibodies are well known to the art.

It will be appreciated that when an animal has raised an immune response to one or more peptides according to the first aspect of the present invention, hyperimmune serum or ascites fluid, for example, can be collected by usual methods. Specific antibodies can be obtained by separation methods known to the art such as precipitation, affinity chromatography, Protein A separation. The separated sera or ascites fluid can be used whole, diluted or as a starting material for separation of one or more peptides according to the first aspect of the present invention.

In one preferred from, the antibodies are detectably labelled. In one preferred form, the label is fluorochrome fluoresein isothiocyanate (FITC). Other labels such as Texas Red, Oregon Green, TRITC, Alexa dyes, allophycocyanin or rhodamine would also be suitable for the present invention. In another preferred form, the antibodies are radioactively labelled.

The assay may be an ELISA assay or radioassay. Other suitable assays utilizing antibodies are well known to the art and include protein chip based matrices. .

In a third aspect, the present invention provides an assay for testing a subject for diabetes or a predisposition to diabetes comprising:

detecting one or more proteins, protein fragments or peptides according to the first aspect of the present invention in a biological sample from the subject; wherein the presence of one or more of the peptides is indicative of diabetes or a predisposition to diabetes.

Preferably, the biological fluid is urine. The urine or other biological fluid can be assayed neat or concentrated or fractionated prior to assaying.

Preferably, the one or more proteins, protein fragments or peptides are detected by the use of an antibody according to second aspect of the present invention.

In a fourth aspect, the present invention provides a kit testing a subject for diabetes or a predisposition to diabetes comprising:

(a) one or more antibodies according to the second aspect of the present invention; and

(b) suitable reagents and diluents for the test.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia prior to development of the present invention.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following examples.

Mode(s) for Carrying Out the Invention

MATERIALS AND METHODS

Before describing the preferred embodiments in detail, the principal of operation of a membrane-based electrophoresis apparatus will first be described. An electric field

or potential applied to ions in solution will cause the ions to move toward one of the electrodes. If the ion has a positive charge, it will move toward the negative electrode (cathode). Conversely, a negatively-charged ion will move toward the positive electrode (anode).

In the apparatus used for present invention, ion-permeable barriers that substantially prevent convective mixing between the adjacent chambers of the apparatus or unit are placed in an electric field and a cell type or population in the sample is selectively transported through an ion-permeable barrier. The particular ion-permeable barriers used will vary for different applications and generally have characteristic average pore sizes and pore size distributions and/or isoelectric points allowing or substantially preventing passage of different components.

Gradiflow™ Apparatus

A number of membrane-based electrophoresis apparatus have been developed by, or in association with, Gradipore Limited, Australia. The apparatus are marketed and used under the name Gradiflow™. In summary, the apparatus typically includes a cartridge which houses a number of membranes forming at least two chambers, cathode and anode in respective electrode chambers connected to a suitable power supply, reservoirs for samples, buffers and electrolytes, pumps for passing samples, buffers and electrolytes, and cooling means to maintain samples, buffers and electrolytes at a required temperature during electrophoresis. The cartridge contains at least three substantially planar membranes disposed and spaced relative to each other to form two chambers through which sample or solvent can be passed. A separation membrane is disposed between two outer membranes (termed restriction membranes as their molecular mass cut-offs are usually smaller than the cut-off of the separation membrane). When the cartridge was installed in the apparatus, the restriction membranes are located adjacent to an electrode. The cartridge is described in AU 738361. Description of membrane-based electrophoresis can be found in US 5039386 and US 5650055 in the name of Gradipore Limited, incorporated herein by reference. An apparatus particularly suitable for use in isoelectric separation applications can be found in WO 02/24314 in the name of The Texas A&M University System and Gradipore Limited, incorporated herein by reference.

The electrophoresis apparatus used in urine separation comprised:

(a) a first electrolyte chamber;

(b) a second electrolyte chamber,

(c) a first sample chamber disposed between the first electrolyte chamber and the second electrolyte chamber;

(d) a second sample chamber disposed adjacent to the first sample chamber disposed and between the first electrolyte chamber and the second electrolyte chamber;

(e) a first ion-permeable barrier disposed between the first sample chamber and the second sample chamber, the first ion-permeable barrier prevents substantial convective mixing of contents of the first and second sample chambers;

(f) a second ion-permeable barrier disposed between the first electrolyte chamber and the first sample chamber, the second ion-permeable barrier prevents substantial convective mixing of contents of the first electrolyte chamber and the first sample chamber;

(g) a third ion-permeable barrier disposed between the second sample chamber and the second electrolyte chamber, the third ion-permeable barrier prevents substantial convective mixing of contents of the second electrolyte chamber and the second sample chamber; and

(h) electrodes disposed in the first and second electrolyte chambers.

The electrophoresis apparatus may further comprise one or more of:

(i) an electrolyte reservoir;

(j) a first sample reservoir and a second sample reservoir;

(k) means for supplying electrolyte from the electrolyte reservoir to the first and second electrolyte chambers; and

(l) means for supplying sample or liquid from at least the first sample reservoir to the first sample chamber, or from the second sample reservoir to the second sample chamber.

The apparatus may comprise:

(m) a first electrolyte reservoir and a second electrolyte reservoir; and

(n) means for supplying electrolyte from the first electrolyte reservoir to the first electrolyte chamber and electrolyte from second electrolyte reservoir to the second electrolyte chamber.

The apparatus may further comprise one or more of:

means for circulating electrolyte from the electrolyte reservoir(s) through the electrolyte chambers forming electrolyte streams in the electrolyte chambers; and

means for circulating contents from each of the first and second sample reservoirs through the respective first and second sample chambers forming first and second sample streams in the respective sample chambers;

means for removing and replacing sample in the first or second sample reservoirs; and

means to maintain temperature of electrolyte and sample solutions.

All ion-permeable barriers were membranes having a characteristic average pore size and pore size distribution.

The electrophoresis apparatus contained a separation unit housing the chambers and ion-permeable barriers which is provided as a cartridge or cassette fluidly connected to the electrolyte reservoir(s) and the sample reservoirs.

In use, the urine sample to be separated was placed in the first or second sample chamber. Electrolyte was placed in the first and second electrolyte chambers. Electrolyte or other liquid can be placed in the first and/or second sample chamber. An electric potential was applied to the electrodes and some urine proteins in the first and/or second sample chamber were caused to move through a diffusion barrier to the second and/or first sample chamber.

Urine samples.

Fifty millilitres of morning urine are collected from Type II diabetic patients and age matched controls. Protein membrane separations were performed with a Gradiflow BF400 apparatus and the protein separation product concentrated 10 times using a standard Acetone-HCl precipitation before freezing at -80°C.

Gradiflow™ separation.

Two protein separations are performed using the Gradiflow BF400 and Tris/EACA/EDTA buffer solution (46.3 g Tris and 5.24 g EACA and 1 mM EDTA in 2 l MilliQ water). The first separation at 250V for 4 hours with a 5-125-5 cartridge (restriction membrane –separation membrane – restriction membrane cut off). The separation product was then used for a second separation, this time with a 5-25-5 kDa cut-off cartridge (250 V, 4 hr). The final product was then concentrated before being stored at -80°C.

Trypsin digestion

Protein was dialysed overnight against water with a 1 kDa cut-off membrane and proteins evaporated to dryness. Samples were resuspended in 1 M Urea, 50 mM

NH_4HCO_3 and 5 mM $CaCl_2$. Trypsin was added at an enzyme to protein ration of 1:50 and the reaction incubated at 37 degrees C for 15h. The peptide digests were evaporated to dryness and resuspended in water to a concentration of 1 ug/ul.

Mass Spectrometry

The peptide mixture was filtered and 1 ug loaded onto a micro C18 precolumn After a 10 min wash the pre-column is switched in line with an analytical column containing C18 RP silica. Peptides were eluted using a linear gradient of H_2O:CH_3CN (95:5, 0.1 % formic acid-buffer A) to H_2O:CH_3CN (40:60, 0.1 % formic acid-buffer B) at 200 nl/min over 30 min. The column was connected via a fused silica capillary to a low volume tee (Upchurch Scientific) where high voltage (2300 V) is applied and a nano electrospray needle is positioned ~ 1 cm from the orifice of a tandem mass spectrometer (either Waters Q-TOF or Applied biosystems Q-Star). Positive ions were generated by electrospray and the mass spectrometer operated in information dependent acquisition mode (IDA). Tandem mass spectra are accumulated for 2 s (m/z 50-2000) and processing scipts are sued to automatically determine peptide sequence.

RESULTS

Peptides sequences were compared between diabetic and non-diabetic patients. Below is a list of peptides occurring in diabetic samples only

Peptide Number	Peptide Sequence:
1	AYIFIDEAHITQALIWLSQR;
2	KEPSQGTTTFAVTSILR;
3	LLDNWDSVTSTFSK;
4	LLIYAVLPTGDVIGDSAK;
5	LLLQQVSLPELPGEYSMK;
6	QEPSQGTTTFAVTSILR;
7	QGLLPVLESFK;
8	TELRPGETLNVNFLLR;
9	VFAIPPSFASIFLTK:
10	WLQGSQELPR.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this fifth day of September 2003

Minomic Pty Ltd

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